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Physiological role of microbodies in the yeast *Trichosporon cutaneum* during growth on ethylamine as the source of energy, carbon and nitrogen

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Abstract. Compartmentation of the metabolism of ethylamine in *Trichosporon cutaneum* X4 was studied in cells, grown on this compound as the sole source of energy, carbon, and nitrogen. Transfer experiments indicated that an amine oxidase is involved in the early metabolism of ethylamine. The synthesis of this enzyme was induced by primary amines and was subject to partial carbon catabolite repression. Repression by ammonium ions was not observed. Adaptation of glucose-grown cells to growth on ethylamine was associated with the development of many microbodies, which developed from already existing organelles present in the inoculum cells and multiplied by division. Cytochemical experiments indicated that the organelles contained amine oxidase and catalase. Therefore, they were considered to play a key role in the metabolism of ethylamine. The physiological significance of the microbodies was investigated by fractionation studies of homogenized protoplasts from ethylamine-grown cells by differential- and sucrose-gradient centrifugation of subcellular organelles. Intact microbodies were only obtained when the isolation procedure was performed at pH 5.8 in the absence of Mg^{2+}-ions. Analysis of the different fractions indicated that the key enzymes of the glyoxylate cycle, namely isocitrate lyase and malate synthase, cosedimented together with catalase and amine oxidase. In addition, activities of malate dehydrogenase, glutamate:oxaloacetate aminotransferase (GOT) and (NAD-dependent) glutamate dehydrogenase were detected in these fractions. Electron microscopy revealed that they mainly contained microbodies. Cytochemical experiments indicated that the above enzymes were all present in the same organelle. These findings suggest that microbodies of ethylamine-grown *T. cutaneum* X4 produce aspartate, so allowing NADH generated in the oxidation of malate by malate dehydrogenase to be quantiatively reoxidized inside the organelles in a series of reactions involving GOT and glutamate dehydrogenase. Aspartase and fumarase were not detected in the microbodies; activities of these two enzymes were present in the cytoplasm.

Key words: Amine metabolism – Microbodies – Amine oxidase – Cytochemistry – Cell fractionation – *Trichosporon cutaneum*

The capacity to use primary amines such as methylamine, ethylamine and butylamine as a source of nitrogen is a widespread property among yeasts (Van Dijken and Bos 1981). Their failure to grow on these compounds as a source of carbon and energy has been explained by the fact that amine oxidase, which is the key enzyme in primary amine catabolism in all yeasts studied so far, is strongly repressed by ammonium ions (Zwart and Harder 1983).

Recently a number of yeast strains have been isolated that are able to use different primary amines as the sole source of carbon and nitrogen (Middelhoven and Hoogkamer-te Niet 1984; Middelhoven et al. 1984b; Veenhuis et al. 1984). Growth of these strains, which belong to the genera *Trichosporon* and *Candida,* was accompanied by increased levels of amine oxidase and catalase in the cells. In yeasts grown on primary amines as a nitrogen source these enzymes are localized in subcellular microbodies (Zwart et al. 1980), defined as peroxisomes (De Duve and Baudhuin 1966). Assimilation of acetaldehyde, the first intermediate generated in the dissimilation of ethylamine, requires the activity of glyoxylate cycle enzymes. During growth of the yeasts *Hansenula polymorpha* and *C. utilis* on ethanol two of the enzymes of this cycle are also localized in microbodies, which are then termed glyoxysomes (Zwart et al. 1983a). In the latter organisms, peroxisomes and glyoxysomes did not exist as separate organelles. Instead, during cultivation of cells in media which required the activity of both glyoxysomal and peroxisomal enzymes for growth, these enzymes were localized in one and the same organelle (Zwart et al. 1983a; Veenhuis and Harder 1986). In view of this we have investigated the metabolism of ethylamine in the newly isolated strain *T. cutaneum* X_4, which is able to grow on this compound as the sole carbon and energy source. Different biochemical and ultrastructural techniques were used to examine (i) the regulation of amine oxidase syntheses, (ii) the significance of microbodies in ethylamine metabolism and (iii) the mode of development of microbodies during growth on ethylamine alone as well as in mixed-substrate cultures together with urate. Urate is also initially metabolized by a microbody-based enzyme and can act as the sole source of carbon and nitrogen in this

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Abbreviations: ABTS, 2,2′-Azino-di-(3-ethylbenzthiazoline sulfonate [6]); DTT, dithiothreitol; GOT, glutamate:oxaloacetate aminotransferase; DTNB, 5,5-dithiobis-2-nitrobenzoate; DAB, diaminobenzidine; BSPT, 2-(2-benzothiazolyl)-3-(4-phthalhydrazidyl)-t-styryl-sH-tetrazolium chloride; PF, convex fracture face; EF, concave fracture face

organism (Middelhoven et al. 1984; Veenhuis et al. 1985). The observed mode of microbody development has been compared with the general mechanisms recently postulated to be involved in the biogenesis of microbodies in *H. polymorpha* and *C. utilis* (Veenhuis and Harder 1986).

Materials and methods

Micro-organisms and cultivation. All experiments were performed with *Trichosporon cutaneum* X_4 isolated from a corn silage as described previously (Veenhuis et al. 1984). The organism was grown in shake flask cultures at 30° C in a basal growth medium which contained per litre: $MgSO_4 \cdot 7\ H_2O$, 0.2 g; NaH_2PO_4, 3 g; K_2HPO_4, 0.75 g; trace elements according to Vishniac and Santer (1957), 1 ml; vitamin solution, 1 ml;and yeast extract, 0.5 g. The vitamin solution contained per 100 ml: 20 mg thiamine, 10 mg riboflavin, 500 mg nicotinic acid, 30 mg para-aminobenzoic acid, 10 mg pyridoxal hydrochloride, 250 mg Ca-pathothenate, 10 mg biotin, and 1 g myo-inositol. Media lacking $(NH_4)_2SO_4$ were supplemented with K_2SO_4, 1 g/l.

After extensive pre-cultivation on glucose/ammonium sulphate, as described previously (Veenhuis et al. 1979) late exponential phase cells were transferred into basal media supplemented with: 0.25% (w/v) glucose; 0.2% (w/v) ammonium sulphate: 0.25% (w/v) glucose; 0.25% (w/v) ethylamine or 0.25% ethylamine, respectively. In addition, cells were grown on 0.25% (w/v) ethylamine in the presence of different concentrations of ammonium sulphate, ranging from 0.001% to 0.2% (w/v). Mixed substrate cultures were grown in a pH and oxygen controlled fermenter (Harder et al. 1974) on 0.25% (w/v) ethylamine and 0.25% (w/v) uric acid. Cells from the stationary growth phase on glucose/ammonium sulphate were used as an inoculum. The pH was kept at 6.7 with 1 N NaOH, oxygen at 80% of air saturation.

Transfer experiments. To cultures of *T. cutaneum* X_4 in the exponential phase of growth on ethylamine either 0.25% (w/v) glucose/0.05% (w/v) ammonium sulphate or 0.25% (v/v) ethanol/0.05% (w/v) ammonium sulphate, respectively, were added. In addition, stationary phase cells were transferred into fresh media with 0.25% (w/v) glucose/0.25% (w/v) ethylamine or 0.25% (w/v) glucose or 0.25% (v/v) ethanol both in the presence of 0.2% (w/v) ammonium sulphate.

Preparations of spheroplasts. Cell wall digestion was tested with the following – commercially available – lytic enzymes: Zymolyase, Helicase, Novozym, chitinase, glucanase, β-glucuronidase or combinations of them, according to the general procedure of Osumi et al. (1975). They all yielded unsatisfactory results, irrespective of pretreatment of the cells with mercaptoethanol or DTT.

Therefore lytic enzymes against cell walls of *T. cutaneum* were raised in *Trichoderma* cultures as follows. Ethylamine-grown cells of *T. cutaneum* X_4 were washed in distilled water, sonified for 10 min at 25 kHz after which the walls were dissolved in 1 N KOH for 1 h at 60° C. After precipitation – by adjusting the pH to 5.0 with 45% (v/v) acetic acid – the precipitate was washed 3 times in distilled water and used as a substrate in the *Trichoderma* cultures. The organism, *Trichoderma viride,* which was kindly provided by Dr H. Sietsma, University of Groningen, The Netherlands, was grown in a liquid medium (Mandels and Reese 1957) that contained 0.3% (w/v) glucose, 0.1% (w/v) peptone, trace elements according to Vishniac and Santer (1957) and approximately 7.5 g/l wet weight cell wall precipitate. After cultivation for 7–8 days a crude enzyme preparation was obtained as described by de Vries (1974).

Protoplasts of *T. cutaneum* X_4 were prepared by treatment of suspensions of whole cells (0.1 g wet weight/ml) with *Trichoderma* enzyme (5 mg/ml) in the presence of helicase (15 mg/ml) during 3 h at 25° C in 0.05 M sodium maleate buffer, pH 5.8, containing 1 mM EDTA and 1.5 M $MgSO_4$ as an osmotic stabilizer.

Cell fractionation. For the isolation of microbodies, protoplasts were harvested by centrifugation and after washing twice in the same buffer, resuspended in 0.05 M maleate acid buffer pH 5.8, containing 1 mM EDTA and 0.2 M $MgSO_4$ and subsequently homogenized in a Potter-Elvehjem homogenizer (Potter 1955) using 10–15 strokes at 200 rpm. Immediately after homogenization the $MgSO_4$ concentration was increased to 0.6 M by adding a calculated volume of 1.5 M $MgSO_4$ and the homogenate differentially centrifuged to remove cell debris and large organelles as described by Zwart et al. (1983a).

The pellet obtained at $30{,}000 \times g$ (P_4) was resuspended in a small volume of 0.05 M maleate acid buffer pH 5.8, containing 1 mM EDTA and 35% (w/w) sucrose and dialysed for 3 h at 4° C against 500 ml of the same buffer in order to remove Mg^{2+}-ions. The buffer was replaced once after 40 min. Subsequently, 2 ml of the dialysed suspension was layered on top of a discontinuous sucrose gradient composed of the maleate buffer with the following sucrose concentrations (w/w): 65% (5 ml), 54% (2.5 ml), 52% (2.5 ml), 50% (2.5 ml), 48% (5 ml), 45% (2.5 ml), 40% (3 ml), and 35% (3 ml). After addition of an overlay containing 20–25% sucrose, the gradient was centrifuged in a Sorvall SS90 vertical rotor at $34{,}500 \times g_{av}$ for 3 h at 4° C. The gradient was harvested by removing samples of 1 ml from the top. These samples were prepared for electron microscopy and tested for enzyme activities.

Enzyme assays. Cell-free extracts were prepared by passing intact cells three times through a French pressure cell (American Instruments Company, Silver Springs, USA) at 0° C and 140,000 kN/m², followed by centrifugation at $30{,}000 \times g$ for 20 min at 4° C to remove unbroken cells and debris.

All enzyme measurements were made in a Perkin-Elmer type 124 spectrophotometer equipped with a thermostated cuvette holder. Enzyme activities were determined at 30° C. The observed rates were linear for at least 5 min and proportional to the amount of extract added.

Amine oxidase was assayed spectrophotometrically as described by Haywood and Large (1981), but absorption was measured at 430 nm. The cuvettes contained in a total volume of 1 ml: 0.05 M Na-phosphate pH 7.0, 5 U/ml peroxidase and 0.5 mg/ml ABTS, 5 mM ethylamine and cell-free extract. Catalase was assayed by the method of Lück (1963). Urate oxidase was measured as described by Bergmeyer et al. (1974c). Isocitrate lyase was assayed as described by Dixon and Kornberg (1959). GOT was assayed by the method of Bergmeyer and Bernt (1974a). Glutamate dehydrogenase was measured by the method of Schmidt (1974). Malate dehydrogenase was assayed by the method

of Bergmeyer and Bernt (1974b). Malate synthase was measured as described by Srere et al. (1963), aspartase as described by Williams and Lartigue (1969) and fumarase as described by Hill and Bradshaw (1968). Acid phosphatase was measured as described by Bergmeyer et al. (1974d). Cytochrome c oxidase was measured as described by Zwart et al. (1983a).

Enzyme activities are expressed as μmol substrate consumed or product formed · min^{-1} mg · $protein^{-1}$ except for catalase which is expressed as $\Delta E240 \cdot min^{-1} \cdot$ mg $protein^{-1}$.

Protein was determined with the method of Bradford (1976) using bovine serum albumin as a standard.

Sucrose concentrations were estimated by measuring the refractive index.

Determination of carbon and nitrogen sources and ammonium. Ethylamine was determined by gas chromatography using a Packard 437 gas chromatograph equipped with a heated flame ionisation detector. A glass column (1.5 m, ∅4 mm) was used, packed with a 28% Penwalt 223 amine packing + 4% KOH on 80/100 Gaschrom R (Alltech associates, Inc.). The column oven temperature was 100° C. The detector temperature was 220° C and the injection temperature 260° C. Nitrogen was used as carrier gas at a flow rate of 40 ml/min. Between measurements the column temperature was occasionally raised to 165° C for approximately 15 min. Uric acid concentrations were determined enzymatically by the method of Scheibe et al. (1974). Ammonium concentrations were measured enzymatically as described Kun and Kearney (1974).

Cytochemical staining. Cytochemical staining procedures for the subcellular localization of catalase, amine oxidase and urate oxidase activity in intact cells or microbody fractions of *T. cutaneum* X_4 were performed by the methods described previously (van Dijken et al. 1975; Veenhuis et al. 1976). Malate synthase, GOT and glutamate dehydrogenase were stained in microbody fractions only. These were prefixed in 4.5% paraformaldehyde in 0.1 M Na-cacodylate buffer pH 7.2 for 30 min at 0° C. Malate synthase activity was stained by the method of Trelease (1975), GOT activity as described by Lee and Torack (1968) and Papadimitrion and Van Duyn (1970). Glutamate dehydrogenase activity was demonstrated by the tetrazolium salt (BSPT) method (Altman 1976).

Immunocytochemistry. Intact cells were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer pH 7.0 for 45 min at 0° C, dehydrated in an ethanol series and embedded in Lowicryl K_4M. Polymerization was at − 35° C by UV-light. Immunogold labelling of amine oxidase was performed on ultrathin sections by the method of Slot and Geuze (1984). Purified amine oxidase, kindly provided by Dr P. J. Large was used for the preparation of antiserum in a rabbit. The quality of the antiserum was tested in an Ouchterlony double diffusion test. Gold particles were prepared by the citrate method described by Frens (1973).

Freeze etching. Cells were incubated for 5 min in 10% (w/v) glycerol, frozen in liquid Freon and freeze-fractured in a Balzer's freeze-etch unit according to the methods described by Moor (1964).

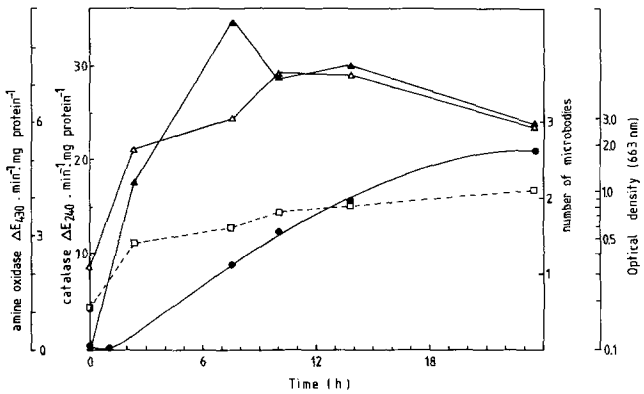


Fig. 1. Growth, enzyme profiles and number of microbodies of *Trichosporon cutaneum* X_4 in batch cultures containing ethylamine as the sole source of energy, carbon and nitrogen. The cultures were inoculated with cells grown in batch culture on glucose/ammonium sulphate ($OD_{663} = 2.3$). ●——● growth (optical density at 633 nm); ▲——▲ amine oxidase; △——△ catalase; □- - - -□ number of microbodies (average per section)

Fixation and postfixation techniques. Whole cells were fixed in 1.5% $KMnO_4$ for 20 min at room temperature. Spheroplasts of microbody fractions were fixed in 4.5% glutaraldehyde or 4.5% paraformaldehyde in 0.1 M Na-cacodylate buffer pH 7.2 for 60 min at 0° C. Postfixation – also after cytochemical staining techniques – was performed in a solution of 1% (w/v) OsO_4 + 2.5% (w/v) $K_2Cr_2O_7$ in the cacodylate buffer for 60 min at 0° C. In case of prefixation in paraformaldehyde, glutaraldehyde (3% v/v) was also added to the postfixation solution. After dehydration in a graded ethanol series, the material was embedded in Epon 812. Ultrathin sections were cut with a diamond knife and examined in a Philips EM 300. Part of the sections was poststained with uranyl acetate and lead citrate (Reynolds 1963). The average number of peroxisomes per cell and their relative volume fraction was determined as described previously (Veenhuis et al. 1979).

Results

Growth and enzyme profiles

After transfer of glucose/ammonium sulphate grown cells of *Trichosporon cutaneum* X_4 into ethylamine containing media, growth started after a lag of approximately 1 h. Concurrent with growth a rapid synthesis of amine oxidase and catalase occurred (Fig. 1). The enzymes reached their maximum activities during mid-exponential growth and gradually decreased thereafter. Amine dehydrogenase activity was not detected in any stage of growth. The optimum pH for growth on ethylamine in batch cultures was 5.8; during growth in such cultures ammonium is excreted in the culture medium. As is evident from Fig. 1 the growth rate in batch cultures (initial $\mu_{max} = 0.23\ h^{-1}$) gradually decreased with time. This phenomenon was especially evident when increased concentrations of ethylamine [up to 1% (w/v)] were present in the cultures (data not shown). This decrease in growth during the mid- and late exponential growth was associated with a change in the mode of vegetative reproduction of the cells: in addition to normal budding cells pseudomycelium and arthrospores were now frequently

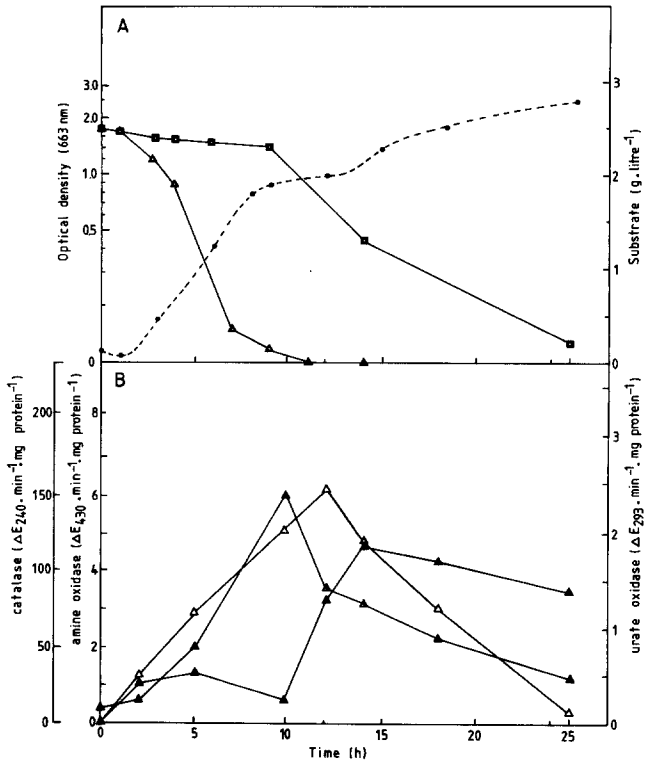


Fig. 2. Growth, uric acid and ethylamine consumption (**A**) and enzyme profiles (**B**) of *T. cutaneum* X_4 in fermenter cultures containing equal amounts (w/v) ethylamine and uric acid. The cultures were inoculated with cells from the exponential phase of growth on glucose/ammonium sulphate. **A** ●----● growth (optical density at 663 nm); ▲——▲ uric acid concentration; ■——■ ethylamine concentration; **B** ▲——▲ amine oxidase; △——△ urate oxidase; △——△ catalase

found (see Fig. 8). The reasons for this phenomenon are unclear; most probably it explains the failure of the organism to grow in chemostat cultures under carbon (ethylamine) limitation. Growth, enzyme profiles and activities were not influenced when ammonium sulphate [up to 0.2% (w/v)] was added to ethylamine-containing media.

During cultivation on a mixture of equal amounts (w/v) of ethylamine and urate diauxic growth was observed (Fig. 2A). The initial growth was associated with rapid synthesis of both urate oxidase and catalase in the cells, whereas amine oxidase remained low (Fig. 2B). Measurements of substrate concentrations showed that this initial growth was almost exclusively at the expense of uric acid; the ethylamine concentration decreased only 8% during the first 10 h of cultivation (Fig. 2A). This was also indicated by the growth rate; the initial μ_{max} of 0.28 h^{-1} was equal to that observed in cultures of *T. cutaneum* X_4 on urate alone. In the lag following depletion of uric acid the activity of amine oxidase increased drastically. At the same time both urate oxidase catalase activities decreased (Fig. 2B). Growth continued after a lag of approximately 2–3 h and the typical growth curve of *T. cutaneum* X_4 at the expense of ethylamine was observed (compare Fig. 1).

Transfer experiments

Transfer of ethylamine-grown cells of *T. cutaneum* X_4 from the early stationary phase of growth into fresh media

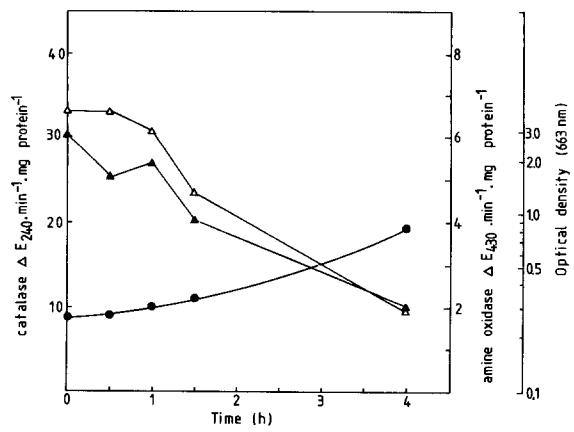


Fig. 3. Growth and enzyme profiles in batch cultures of *T. cutaneum* X_4 after transfer of cells from the stationary growth phase on ethylamine into fresh media with glucose/ammonium sulphate. Symbols as in Fig. 1

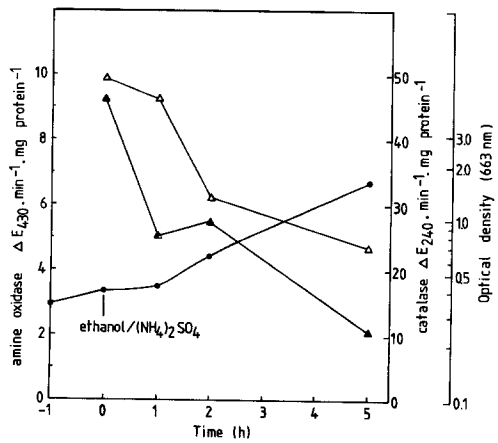
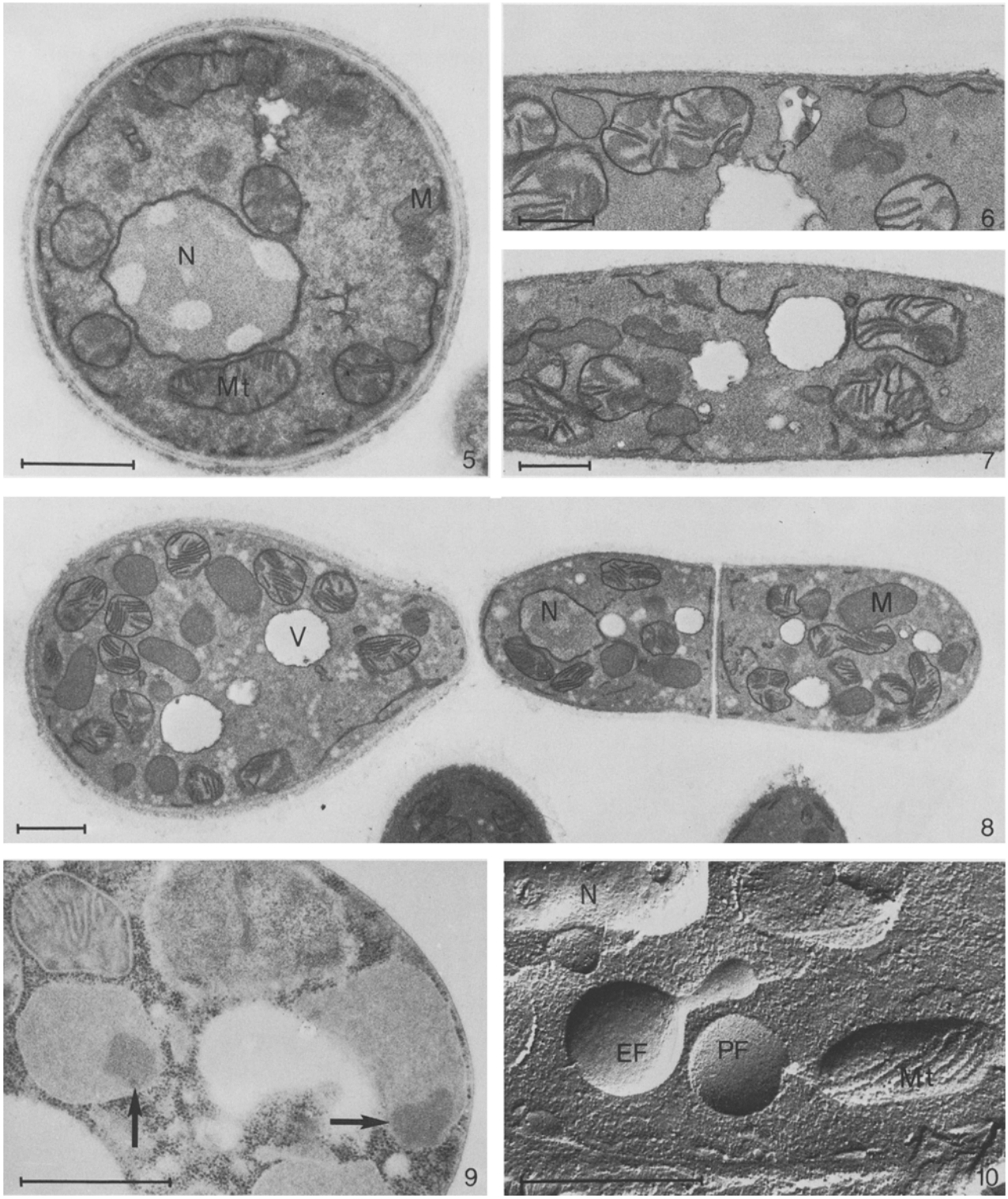


Fig. 4. Growth and enzyme profiles of *T. cutaneum* X_4 after addition of ethanol/ammonium sulphate to cultures growing exponentially on ethylamine as the sole source of carbon and nitrogen. Symbols as in Fig. 1

containing glucose/ammonium during the first hours was associated with a decrease in both amine oxidase and catalase activities (Fig. 3). Inactivation was not observed; the decrease in enzyme activity could be accounted for by dilution of enzyme protein as a result of growth. Transfer of cells into glucose/ethylamine containing media also resulted in a slight decrease in amine oxidase and catalase activities. After 90 min of incubation the specific activity of amine oxidase in the cultures had decreased by approximately 16% and that of catalase by 21%, indicating that the synthesis of both enzymes is only partially repressed under these conditions. In contrast, when ethylamine-grown cells were transferred into ethanol/ammonium sulfate media a rapid inactivation of amine oxidase and catalase activities was observed. Similar patterns of enzyme inactivation were found when ethanol was added to cultures of *T. cutaneum* X_4 growing exponentially on ethylamine (Fig. 4).

Electron microscopy

Glucose/ ammonium suphate-grown cells of *T. cutaneum* X_4 generally contained a few small microbodies, which mea-



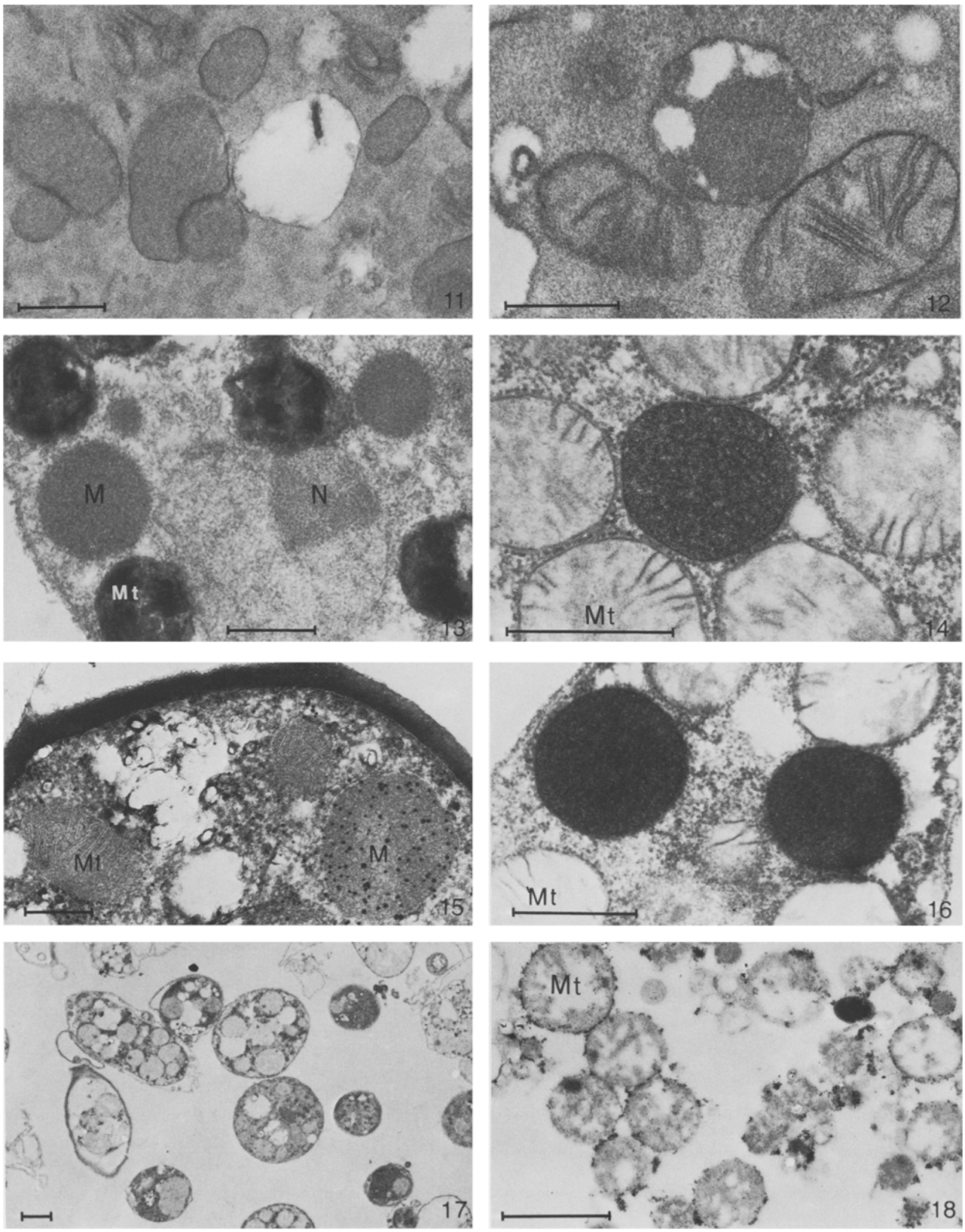
Electron micrographs: The micrographs are taken from $KMnO_4$-fixed cells of *T. cutaneum* X_4, grown on ethylamine as the sole carbon and nitrogen source, unless otherwise indicated.
Abbreviations: L lipid droplet; *M* microbody; *Mt* mitochondrion; *N* nucleus; *V* vacuole.
The marker represents 0.5 μM

Fig. 5. Survey of glucose/ammonium sulphate-grown cells in which two profiles of the small microbodies, characteristic for these cells, are shown

Figs. 6 and 7. Details of cells showing proliferation and distribution of microbodies in the early stages of exponential growth on ethylamine. The cultures were inoculated with glucose/ammonium sulphate-grown cells at $OD_{663} = 1.0$ (compare Fig. 5). **Fig. 6.** 2 h of cultivation; $OD_{663} = 0.125$; **Fig. 7.** 6 h of cultivation; $OD_{663} = 0.3$

Fig. 8. Survey of cells typically observed in batch cultures during the mid-exponential growth phase ($OD_{663} = 0.8$), when pseudomycelium is frequently formed. Both the normal vegetative mothercell (left) and the pseudomycelial cells contain several microbody profiles

Figs. 9 and 10. Details of cells showing the presence of a dense core in the microbody matrix after glutaraldehyde-OsO_4/$K_2Cr_2O_7$ fixation (Fig. 9). After freeze-etching (Fig. 10) the EF (concave fracture face) shows a typical smooth appearance whereas in the PF (convex fracture face) a number of small particles are observed (nomenclature after Branton et al. 1975)



Figs. 11 – 18

sured up to 0.2 μm. (Fig. 5). Transfer of such cells into ethylamine-containing media resulted in an increase in both number and volume fraction of the microbodies. Their number tripled in the first 5 h of cultivation. The organelles were scattered throughout the cytoplasm; clusters of microbodies were not observed (Fig. 6, 7). During subsequent cultivation the number and size of microbodies slowly increased (Fig. 8). In thin sections of $KMnO_4$-fixed cells they were generally observed in close association with mitochondrial profiles. In cultures from the early stationary phase of growth the organelles measured up to 0.7 μm and generally contained a dense core (Fig. 9). Their volume fraction then amounted to approximately 4% of the cytoplasmic volume. After freeze etch experiments, performed in order to investigate the substructure of the peroxisomal membranes, the PF showed a smooth appearance, whereas on the EF a relatively low number of small particles were observed (Fig. 10). Similar patterns of microbody proliferation – both in qualitative and quantitative terms – were observed when different concentrations of ammonium sulphate were added to the ethylamine-containing media.

During the first hours of growth in a mixture of equal amounts (w/v) of ethylamine and urate proliferation of clusters of microbodies was observed. This is characteristic for growth of *T. cutaneum* strains on uric acid as the sole source of carbon and nitrogen (Veenhuis et al. 1985) (Fig. 11). However, during adaptation of cells to growth on ethylamine these clusters gradually disappeared and the microbodies were again randomly distributed throughout the cytoplasm after growth on ethylamine had resumed.

After transfer of ethylamine-grown cells into fresh glucose/ammonium sulphate- or glucose/ethylamine-containing media, degradation of microbodies was not observed. Similar results were obtained when glucose was added to cultures growing exponentially on ethylamine. However, the loss of amine oxidase activity observed after transfer of cells into ethanol/ammonium sulphate media was associated with a decrease in number and volume fraction of the microbodies. Within 2 h after the transfer of cells, the volume fraction of the microbodies had decreased to 40% and their number to 80% of the original values. Thin sectioning revealed that part of the organelles were degraded by means of an autophagic process (Fig. 12), similar to that described previously for the glucose-induced degradation of peroxisomes in methanol-grown cells of *Hansenula polymorpha* (Veenhuis et al. 1983a). For this reason the selective inactivation of amine oxidase under ethanol excess conditions must be considered a case of degradative inactivation (Switzer 1977). As already indicated by the morphometric data especially the larger microbodies were subject to degradation.

Cytochemistry

The presence of catalase activity in microbodies of ethylamine-grown cells was demonstrated after incubations with DAB and H_2O_2 (Fig. 13). Amine oxidase activity was demonstrated by aerobic incubations with $CeCl_3$ and ethylamine (Fig. 14). The cytochemical experiments did not allow us to discriminate whether staining of the mitochondrial cristae observed in the latter experiments was fully due to peroxidase activities (Veenhuis et al. 1976) or that amine oxidase activity was also present in mitochondria. For this reason immunocytochemical experiments were performed. After immunogold staining carried out on ultrathin sections of K_4M-embedded cells using antibodies against amine oxidase, gold particles were largely confined to the microbody matrix (Fig. 15). Specific labeling of mitochondria was not observed.

Experiments on cells from mixed-substrate cultures on ethylamine/urate taken from the first lag phase ($t = 12$, Fig. 2A) during the adaptation to growth on ethylamine revealed that in such cells the microbodies were all positively stained for amine oxidase (Fig. 16) or urate oxidase activity, respectively. These results indicated that at this stage of cultivation amine oxidase and urate oxidase were both present in one and the same organelle.

Cell fractionation experiments

The subcellular localization of the different enzymes, thought to be involved in ethylamine metabolism, was investigated by cell fractionation studies which involved homogenisation of protoplasts followed by differential – and sucrose gradient centrifugation. Fractionation studies using protoplast homogenates of cells of *T. cutaneum* X_4 from the exponential growth phase ($OD_{663} = 0.9$), carried out at

Fig. 11. Detail of a cell from the initial exponential growth phase on ethylamine and uric acid ($OD_{663} = 0.5$) showing the clusters of microbodies typical for growth on uric acid

Fig. 12. Detail of a cell grown on ethylamine, 60 min after the addition of ethanol/ammonium sulphate to the culture, showing degradation of a microbody

Figs. 13 and 14. Details of spheroplasts, showing the presence of catalase (Fig. 13) and amine oxidase activity (Fig. 14) in the microbody matrix. Catalase was demonstrated with DAB and H_2O_2, amine oxidase activity with $CeCl_3$ and ethylamine (aldehyde – $OsO_4/K_2Cr_2O_7$)

Fig. 15. Detail of a spheroplast embedded in K_4M showing the labeling pattern after immunocytochemical staining of amine oxidase by the protein A-gold method. Using antibodies against amine oxidase specific labeling is confined to the microbody-matrix (glutaraldehyde-uranyl acetate)

Fig. 16. Detail of a spheroplast of a cell grown on a mixture of ethylamine and uric acid, taken from the lag phase (at T = 12; Fig. 2A) during adaptation of cells from growth on uric acid to growth on ethylamine. All microbodies were positively stained after incubation with $CeCl_3$ and ethylamine (aldehyde – $OsO_4/K_2Cr_2O_7$)

Figs. 17 and 18. Survey of the $5{,}000 \times g$ pellet (P_1) (**Fig. 17**) and the $12{,}500 \times g$ pellet (P_3) (**Fig. 18**) obtained after differential centrifugation of cell homogenates at pH 5.8. In P_1 intact and partly disrupted cells are observed, in P_3 mitochondria are dominant (glutaraldehyde – $OsO_4/K_2Cr_2O_7$)

Table 1. Influence of pH and Mg^{2+}-ions on the recovery of the microbody matrix enzymes catalase, amine oxidase and isocitrate lyase in the microbody-peak fractions after centrifugation of the 30,000 × *g* pellet (P4) (obtained by differential centrifugation of homogenized protoplasts of *Trichosporon cutaneum* X_4) on a discontinuous sucrose gradient. Cells were grown on ethylamine as the sole source of carbon and nitrogen. The data are expressed as percentage of the activity originally present in P4

Sucrose gradient centrifugation at	Percentage of the total activity present in the microbody-peak fraction		
	Catalase	Amine oxidase	Isocitrate lyase
pH 5.8 + $MgSO_4$	80	80	86
pH 7.5 + sorbitol	6	6	–
pH 5.8 – $MgSO_4$ (dialysed)	63	59	65

– Not determined

Table 2. Specific enzyme activities in cell free extracts of *T. cutaneum* X_4 grown in batch cultures on glucose/ammonium sulphate or on ethylamine as the sole carbon and nitrogen source. Cells were harvested from the late-exponential growth phase

Growth conditions	Specific activity of				
	Glutamate dehydrogenase				
	NAD	NADP	GOT	Aspar-tase	Fumar-ase
Glucose/ ammoniumsulphate (OD_{663} = 2.4)	3.060	0.341	1.745	0.027	0.937
Ethylamine (OD_{663} = 1.3)	0.509	0.368	1.688	0.040	0.118

Enzyme activities are expressed as U/min^{-1} mg $protein^{-1}$

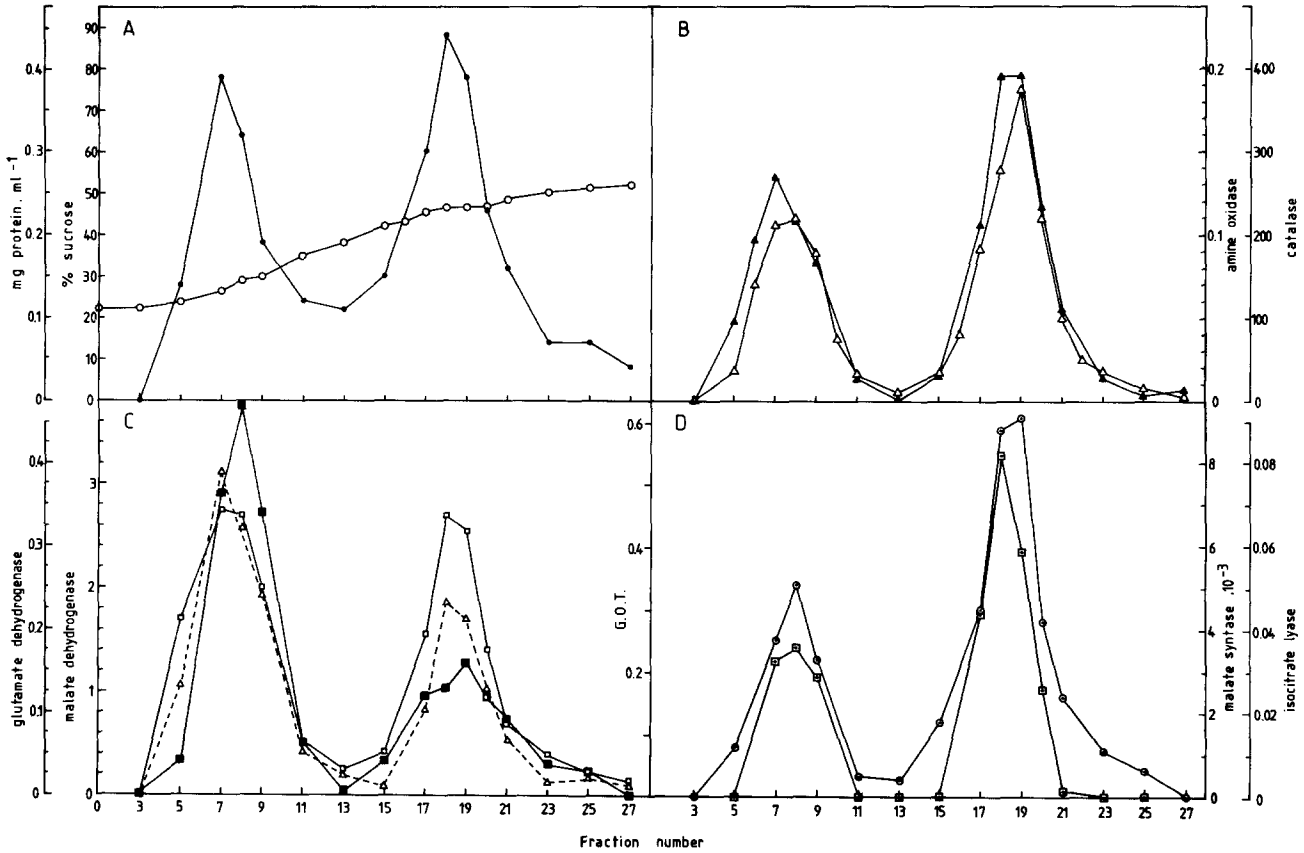
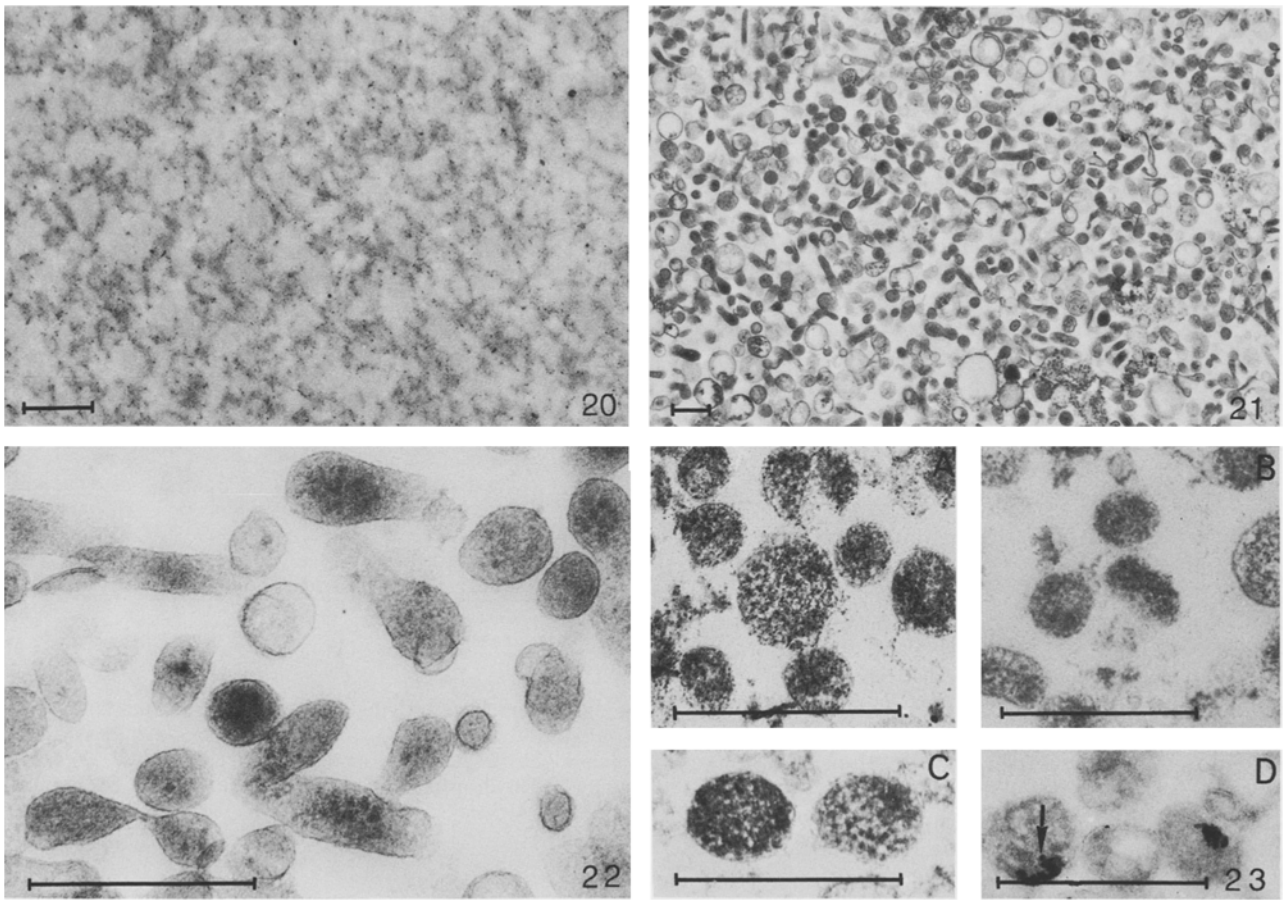


Fig. 19. Distribution of different enzymes after sucrose gradient centrifugation of a fraction enriched in microbodies, obtained by differential centrifugation of homogenized protoplasts of *T. cutaneum* X_4 (for details see Materials and methods section). The cells were harvested from batch cultures in the exponential growth phase on ethylamine as the carbon and nitrogen source (OD_{663} = 1.2). ○——○ Sucrose concentration; ●——● protein; ▲——▲ amine oxidase; △——△ catalase; △- - -△ glutamate oxaloacetate amino transferase; ■——■ (NAD) glutamate dehydrogenase; □——□ malate dehydrogenase; ⊙——⊙ malate synthase; ⊡——⊡ isocitrate lyase

pH 5.8 in the presence of $MgSO_4$ as an osmotic stabilizer revealed that approximately 90% of the amine oxidase activity that was present in the crude homogenates was ultimately sedimented. Generally 45 – 50% of the amine oxidase, originally present in the crude homogenate was recovered in the first (5,000 × *g*) pellet, whereas approximately 20 – 25% was detected in fraction P_4 (30,000 × *g* pellet). In the latter pellets the activity of cytochrome c oxidase was generally undetectably low. The activity of this enzyme was largely confined to the fractions P_1 (5,000 × *g* pellet) and P_3 (12,500 × *g* pellet). The biochemical results were confirmed by electron microscopical analysis of thin sections prepared from embedded samples of the various fractions obtained during differential centrifugation. P_1 contained cell debris



Figs. 20–22. Electron micrographs of material obtained after high speed centrifugation of the top layer (**Fig. 20**) and of isolated microbodies (**Fig. 21**) after sucrose gradient centrifugation of the dialysed 30,000 × *g* pellet (P_4) after fixation with glutaraldehyde – $OsO_4/K_2Cr_2O_7$. A high magnification of isolated microbodies is shown in **Fig. 22**

Fig. 23. shows isolated microbodies, positively stained for malate synthase **A,** catalase **B,** (NAD) glutamate dehydrogenase **C,** and glutamate oxaloacetate aminotransferase activity **D** (prefixation: formaldehyde; postfixation: glutaraldehyde – $OsO_4/K_2Cr_2O_7$)

and virtually intact cells together with intact or partially lysed spheroplasts, probably due to incomplete protoplast formation and homogenization (Fig. 17). P_3 mainly consisted of intact mitochondria and relatively few microbodies, membraneous vesicles and remnants of protoplasts (Fig. 18), whereas in P_4 microbodies were highly dominant. This fraction also contained few unidentified membranous structures, but mitochondrial profiles were generally not observed. P_4 was ultimately subjected to density centrifugation on a discontinuous sucrose gradient. Biochemical experiments indicated that this pellet is highly enriched in amine oxidase; specific activities of this enzyme in P_4 were at least ten fold that of the original cell homogenate. The distribution of the different enzymes after sucrose gradient centrifugation was strongly dependent on the nature and pH of the buffer, the osmotic stabilizer and the presence of Mg^{2+}-ions. For instance, experiments performed at pH 7.5 in the presence of sorbitol resulted in severe leakage of microbody matrix enzymes (Table 1). Best results were obtained at pH 5.8 in the presence of Mg^{2+}-ions where 80–85% of the amine oxidase activities of P_4 were ultimately recovered in the microbody-peak fractions (Table 1). However, under these conditions the microbodies and the other subcellular components were hardly separated. Electron microscopy of these peak fractions indicated that this was most probably due to coagulation of these organelles together with remnants of the cell wall. Coagulation was prevented when P_4 was dialysed in order to remove Mg^{2+}-ions prior to sucrose gradient centrifugation. After sucrose gradient centrifugation of this P_4 a distinct narrow band was obtained at approximately 46.5% sucrose. As shown in Table 1, 60–65% of the activities of catalase and amine oxidase, originally present in P_4, was recovered in the microbody-peak fractions (fractions 16–21). Bulk of the remaining activity was detected in the top layer (fractions 5–11), indicating that part of the organelles was destroyed under these conditions. This was confirmed by electron microscopy. In thin sections prepared from the pooled fractions 5–11 intact organelles were not found; instead aggregates of protein molecules and remnants of membranes were observed (Fig. 20). Typical patterns of protein and enzyme activities after sucrose-gradient centrifugation are shown in Fig. 19A–D. As is evident from this figure, the peroxisomal enzymes catalase and amine oxidase (Fig. 19B) cosedimented with key enzymes of the glyoxylate cycle, isocitrate lyase and malate synthase (Fig. 19D). Activities of malate dehydrogenase and glutamate-oxaloacetate aminotransferase (GOT) and NAD-dependent glutamate dehydrogenase were also present in these fractions (Fig. 19C). NADP-dependent glutamate dehydrogenase

was invariably absent; however, activities of this enzyme were present in cell-free extracts prepared from ethylamine-grown cells. Similar results were obtained with aspartase and fumarase. Both enzymes were absent in the microbody fractions but present in cell-free extracts. Activities of these enzymes were also detected in glucose/ammonium sulphate-grown cells (Table 2). Acid phosphatase activity, a vacuolar marker enzyme in yeasts (Wiemken et al. 1979; Veenhuis et al. 1980), was not detected in the microbody-peak fractions and was extremely low in the top layer. The bulk of the acid phosphatase activity (approximately 80%) was detected in the supernatant of the 30,000 × *g* pellet (S 4), whereas 15% was present in the 5,000 × *g* pellet.

Electron microscopic observations revealed that the band at 46.5% sucrose almost exclusively contained microbodies (Fig. 21–22). The presence of amine oxidase, malate synthase, catalase, (NAD)glutamate dehydrogenase and GOT in these organelles was confirmed cytochemically (Fig. 23 A–D).

Discussion

We have shown that growth of *Trichosporon cutaneum* X_4 at the expense of ethylamine as a carbon and nitrogen source is associated with enhanced levels of amine oxidase and catalase. These enzymes have previously been encountered as key enzymes in other yeasts when primary amines were the nitrogen source (Zwart et al. 1980; Veenhuis et al. 1981; Zwart and Harder 1983). The inability of the latter organisms to utilize primary amines as a carbon source was investigated in detail by Zwart and Harder (1983), who showed that in *Candida utilis* and *Hansenula polymorpha* amine oxidase was strongly repressed by ammonium ions and derepressed under nitrogen limitation. During growth of these organisms on ethylamine as the nitrogen source, the amount of amine utilized was just sufficient to account for growth and satisfy the cellular nitrogen requirement; the rate of amine oxidation was determined by intracellular concentrations of ammonium via repression/derepression of amine oxidase synthesis. As expected, in *T. cutaneum* X_4 this type of control was not found. Growth and amine oxidase activity was not influenced by the presence of ammonium ions in the culture medium; the synthesis of amine oxidase was induced by primary amines and subject to partical carbon catabolite repression.

At the subcellular level ethylamine-grown cells of *T. cutaneum* X_4 were characterized by the presence of many microbodies. Our results provide evidence that these organelles are the sole sites of cytoplasmic amine oxidase and catalase activity. They are therefore involved in the initial metabolism of this combined carbon and nitrogen source (Zwart et al. 1983b).

Their physiological significance was investigated using cell fractionation procedures. These experiments indicated that, as was observed in methylotrophic yeasts (Goodman et al. 1984), the stability of microbodies from *T. cutaneum* X_4 was greatly enhanced at lower pH values. A further stabilization was obtained when Mg^{2+}-ions were present.

Microbodies, isolated from ethylamine-grown *T. cutaneum* X_4, contained the same matrix enzymes as those obtained from *C. utilis* after growth on this compound as a nitrogen source (Zwart et al. 1980; Zwart 1983) namely amine oxidase, catalase, isocitrate lyase, malate dehydrogenase, and GOT. In addition activity of NAD-dependent glutamate dehydrogenase was detected in the present study.

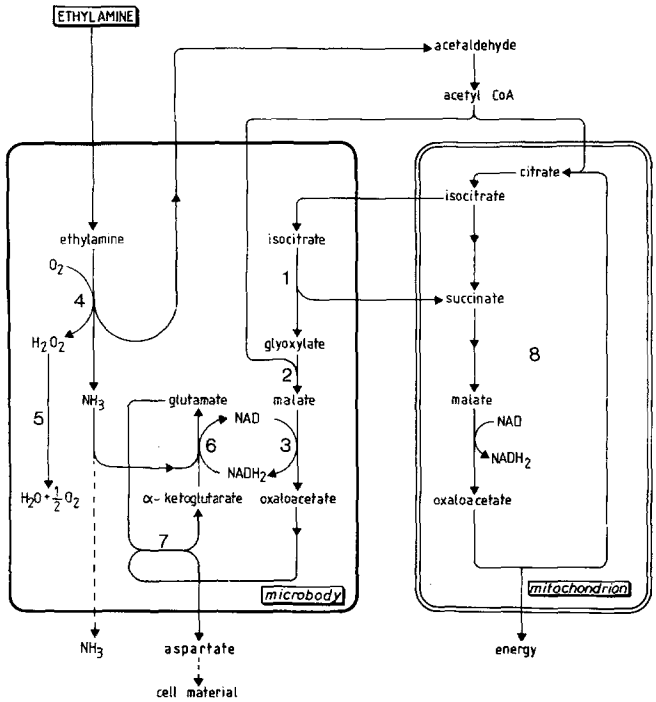


Fig. 24. Metabolism of ethylamine by microbodies and mitochondria in *T. cutaneum* X_4. *1* isocitrate layse; *2* malate synthase; *3* malate dehydrogenase; *4* amine oxidase; *5* catalase; *6* (NAD) glutamate dehydrogenase; *7* glutamate:oxaloacetate aminotransferase; *8* TCA cycle

This enzyme has not previously been encountered in yeast microbodies. Its presence may also explain the significance of malate dehydrogenase and GOT in these organelles, which was unclear until now (Zwart et al. 1983a). In plants these enzymes are thought to be involved in a shuttle mechanism for NADH transport across the microbody membrane (Tolbert 1981). In microbodies of ethylamine-grown *T. cutaneum* X_4 NADH produced by malate dehydrogenase is quantitatively reoxidized in a series of reactions in which glutamate dehydrogenase and GOT play an essential role (Fig. 24). The aspartate thus produced is thought to leave the microbody and is further metabolized via aspartase and fumarase which are most likely present in the cytoplasm of ethylamine-grown *T. cutaneum* X_4. After its conversion to malate and subsequently to oxaloacetate by cytoplasmic malate dehydrogenase it is available for gluconeogenis and other biosynthetic processes. In this scheme an important role is played by cytoplasmic aspartase, an enzyme that is quite uncommon in yeasts (Thomulka and Moat 1972; Zwart et al. 1983c).

Our results also indicate that in ethylamine-grown *T. cutaneum* X_4 cells the different enzymes mentioned above were all localized in the same organelles. The relatively high leak especially of glutamate dehydrogenase, malate dehydrogenase and GOT observed after sucrose gradient centrifugation of the 30,000 × *g* pellet may point to the presence of different types of organelle – which has for instance been suggested in the fungus *Neurospora crassa* (Theimer et al. 1978) – and the leak explained by differences in fragility/stability. However, this possibility is clearly ruled out by the results of cytochemical staining experiments on isolated microbodies. Therefore, as described for *C. utilis* and *H. polymorpha* (Zwart et al. 1983a; Veenhuis and Harder

1986), the microbodies in *T. cutaneum* X_4 constitute one class of organelles. They develop from already existing organelles and their ultimate physiological function(s) – peroxisomal, glyoxysomal, biosynthetic or intermediate – is a reflection of environmental conditions which prevail during growth.

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